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NLRX1 alleviates lipopolysaccharide-induced apoptosis and inflammation in chondrocytes by suppressing the activation of NF-κB signaling

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ABSTRACT

Osteoarthritis (OA) is a chronic debilitating disease characterized by joint degeneration. Excessive chondrocyte apoptosis and inflammation contributes to articular cartilage destruction in OA pathology. Nucleotide-binding oligomerization domain (NOD)-like receptor X1 (NLRX1) has emerged as a critical regulator of inflammation that participates in the pathology of diverse diseases. To date, little is known about the role of NLRX1 in OA. In the present study, we aimed to explore the function of NLRX1 in lipopolysaccharide (LPS)-induced injury in chondrocytes, an *in vitro* model of OA. NLRX1 mRNA was detected by quantitative polymerase chain reaction (qPCR) analysis. Protein expression of NLRX1, phosphorylated IκB kinase β (IKKβ), and phosphorylated nuclear factor-κB (NF-κB) p65 were examined by western blot. Cell viability was assessed by the MTT assay. Cell apoptosis was evaluated by measuring caspase-3 activity. Cytokine release was assessed by enzyme-linked immunosorbent assay (ELISA). NF-κB signaling activation was analyzed with a luciferase reporter assay. Herein, our results revealed that NLRX1 expression was markedly decreased in LPS-treated chondrocytes. Functional experiments demonstrated that NLRX1 overexpression significantly improved cell viability and attenuated LPS-treated chondrocyte apoptosis and inflammation, while NLRX1 silencing caused the opposite effects. Moreover, our results showed that NLRX1 regulated LPS-induced NF-κB signaling activation. Notably, NF-κB signaling inhibition significantly reversed the NLRX1-knockdown-mediated enhanced effects on LPS-induced apoptosis and inflammation. Overall, these results demonstrate that NLRX1 alleviates LPS-induced apoptosis and inflammation in chondrocytes by negatively regulating NF-κB signaling, results that indicate an anti-inflammatory role for NLRX1 in OA. Our findings suggest that NLRX1 may serve as a potential therapeutic target for OA.

1. Introduction

Osteoarthritis (OA), characterized by joint degeneration, is a chronic debilitating disease that typically affects the spine, hands, knees, hips, and feet [1]. Articular cartilage destruction is the most prominent feature in OA [2,3]. Chondrocytes, the primary cells in articular cartilage, are responsible for its synthesis and degradation [4,5]. Excessive chondrocyte apoptosis and inflammation contributes to cartilage degeneration [6,7]. Inflammatory cytokines in the synovial fluid triggered by pathogenic stimuli shift chondrocytes to a catabolic phenotype, and this change contributes to OA pathology [8]. Therefore, a better understanding of the molecular mechanism that underlies chondrocyte-related apoptosis and inflammation may provide novel insights into the development of promising therapeutic options for OA.

OA pathophysiology involves the excessive expression of pro-inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) [9]. The expression of these pro-inflammatory cytokines is controlled by the transcription factor nuclear factor-κB (NF-κB). Under unstimulated conditions, NF-κB is sequestered in the cytosol by binding with inhibitor of NF-κB (IκB). Upon stimulation, IκB is phosphorylated by IκB kinases (IKKs), which targets it for degradation, and NF-κB is released and translocated from the cytoplasm into the nucleus to activate the transcription of pro-inflammatory cytokines [10]. NF-κB plays an important role in OA pathology [11]. Activation of NF-κB signaling contributes to the induction of articular cartilage destruction [12–15]. Therefore, NF-κB signaling has been suggested as a potential target for OA therapy. Various components regulate NF-κB signaling activation. However, the regulatory

Abbreviations: OA, osteoarthritis; NLRX1, nucleotide-binding oligomerization domain-like receptor X1; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IL, interleukin; TNF-α, tumor necrosis factor-α; IKK, IκB kinase; qPCR, quantitative polymerase chain reaction

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mechanisms of NF-κB signaling remain only partially understood.

Nucleotide-binding oligomerization domain (NOD)-like receptor X1 (NLRX1) is a member of the NLR family that plays a crucial role in regulating inflammation [16]. NLRX1 is a mitochondrial protein that regulates diverse cellular processes and participates in various pathological processes [17,18]. NLRX1 is a potential tumor suppressor that represses the growth and metastasis of various tumors [19–21]. NLRX1 regulates innate immunity by restricting viral and bacterial infections [22–24]. NLRX1 suppression alleviates high fat diet-induced hyperglycemia and hepatic steatosis in mice [25,26]. Accumulating evidence suggests that NLRX1 protects various cells against apoptosis by inhibiting inflammation and oxidative stress [27–29]. Therefore, the role of NLRX1 has attracted considerable interest, namely regarding its critical role in pathophysiology.

NLRX1 has emerged as a crucial regulator of NF-κB signaling [30,31]. Considering that NF-κB is a critical regulator of OA, we hypothesized that NLRX1 may participate in OA through regulating NF-κB signaling. This study aimed to investigate the potential role of NLRX1 in lipopolysaccharide (LPS)-induced apoptosis and inflammation in chondrocytes, an *in vitro* model of OA.

2. Materials and methods

2.1. Chondrocyte isolation, culture, and treatment

Articular chondrocytes were isolated from mouse joints as described previously [32]. Briefly, articular cartilage was isolated from femoral condyles and tibial plateaus of newborn mice. Tissues were washed with sterile phosphate buffered saline (PBS), cut into small pieces, and digested with 0.2% type II collagenase. Cells were then seeded in a new Petri dish with Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA). The dissociated cells were collected and cultured in DMEM with 10% fetal bovine serum (FBS) and a 1% penicillin and streptomycin mix. Cells were maintained at 37 °C in a humidified incubator with 5% CO_2. For induction of apoptosis and inflammation, chondrocytes were treated with 10 μg/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 6 h.

2.2. Cell transfection

The short hairpin RNA (shRNA) sequences that targeted NLRX1 were subcloned into a pLKO.1 vector to generate the NLRX1 shRNA expression vector. The NLRX1 cDNA sequence was subcloned into a pcDNA3.1 vector to construct the NLRX1 expression vector. The constructs were transfected into cells using Lipofectamine 3000 Reagent (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol.

2.3. RNA extraction and quantitative polymerase chain reaction (qPCR) assays

Total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen) as per the manufacturer's manual. Total RNA was converted into complementary DNA (cDNA) using TaqMan Reverse Transcription Reagents (Invitrogen). qPCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with appropriate primers. The primer sequences were: NLRX1 forward (5′-TAGGGCCTTTATCCGTTACCA-3′) and reverse (5′-TAAACC ACTCGGTGAGGTTCC-3′) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward (5′-TGGATTTGGACGCATTGGTC-3′) and reverse (5′-TTTGCACTGGTACGTGTTGAT-3′). qPCR was performed using an Applied Biosystems AB7500 Real-Time PCR system using the following thermal cycling conditions: 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. GAPDH served as a control housekeeping gene for gene expression normalization using the $2^{-\Delta\Delta Ct}$ method [33].

2.4. Western blot analysis

Proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer that contained a protease inhibitor cocktail (Sigma-Aldrich). Protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). Equal protein amounts were loaded on a 10% sodium dodecyl sulfate polyacrylamide gel and separated by electrophoresis. Next, the separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, and the membrane was immersed in 5% skim milk in Tris-buffered saline-Tween 20 (TBST) for 1 h at room temperature. Subsequently, the membrane was incubated with primary antibodies specific for NLRX1, p-IKKβ, NF-κB p-p65, or GAPDH (Abcam, Cambridge, MA, USA). After overnight incubation at 4 °C, the membrane was washed three times with TBST. Thereafter, the membrane was incubated with the appropriate horseradish peroxidase (HRP)-labeled secondary antibodies for 1 h at room temperature. Protein bands were visualized with an enhanced chemiluminescence detection system.

2.5. Cell viability assay

Cell viability was measured with the MTT Cell Viability Assay Kit (RiboBio Co., Ltd., Guangzhou, China) as per the manufacturer's instructions. Briefly, cells were plated onto 96-well plates at 5×10^3 cells/well and transfected with the NLRX1 shRNA or expression vector for 48 h and then exposed to LPS for 6 h. Subsequently, the medium was discarded and 100 μL of fresh medium that contained 10 μL of MTT solution (5 mg/mL) was added to each well. After culturing for 4 h at 37 °C in the dark, 100 μL of formazan solubilization buffer was added to each well. After the formazan was dissolved, the optical density value at 570 nm was determined with a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

2.6. Caspase-3 activity assay

Caspase-3 activity assay was measured using a Caspase-3 Activity Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocols. In brief, cells were plated onto 48-well plates at 1×10^5 cells/well and transfected with the NLRX1 shRNA or expression vector for 48 h and then exposed to LPS for 6 h. Next, 2×10^6 cells were harvested and lysed with 100 μL of lysis buffer. The supernatants were harvested by centrifugation at 16,000*g* at 4 °C for 15 min. Subsequently, approximately 50 μL of the supernatants from each sample were incubated with 10 μL of Ac-DEVD-pNA and 40 μL of reaction buffer. After incubation for 2 h at 37 °C, the optical density value at 405 nm was measured with a microplate reader.

2.7. Cytokine release assay

Cell culture supernatants were collected at the indicated times after treatment and analyzed for cytokine concentration with commercial enzyme-linked immunosorbent assay (ELISA) kits for IL-1β (sensitivity: 4.8 pg/mL; assay range: 12.5–800 pg/mL), IL-6 (sensitivity: 1.8 pg/mL; assay range: 7.8–500 pg/mL), and TNF-α (sensitivity: 7.21 pg/mL; assay range: 10.9–1000 pg/mL; R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocols.

2.8. Luciferase reporter assay

Cells were seeded in a 24-well plate at 2×10^5 cells/well and co-transfected with the pNF-κB-luc reporter vector (Beyotime Institute of Biotechnology) that contained NF-κB binding motifs, pRL-TK Renilla luciferase vector, and the NLRX1 shRNA or expression vector using Lipofectamine 3000 (Invitrogen). After transfection for 48 h, cells were treated with LPS for 6 h. Thereafter, cellular luciferase activities were

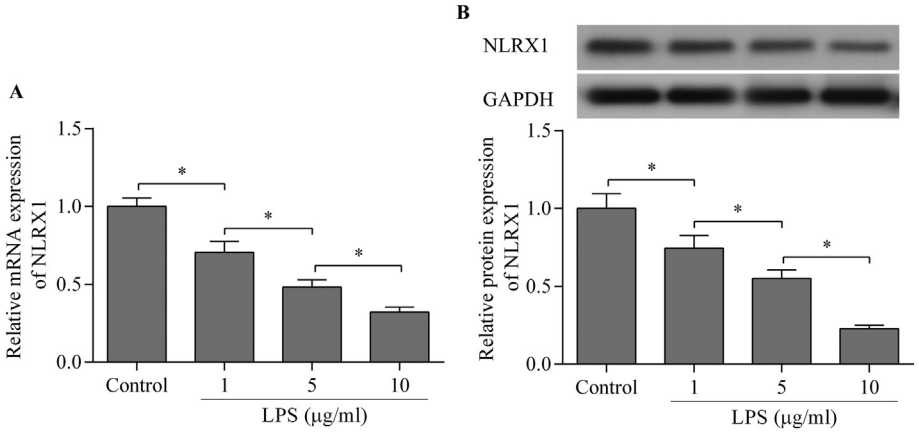

Fig. 1. NLRX1 expression was decreased in chondrocytes after LPS treatment. (A) Relative NLRX1 mRNA expression in chondrocytes exposed to LPS was examined by qPCR. (B) NLRX1 protein expression in chondrocytes exposed to LPS was detected by western blot. Chondrocytes were stimulated with 1, 5, or 10 μg/mL of LPS for 6 h. Cells treated with vehicle were used as control. *$p < 0.05$ *versus* control.

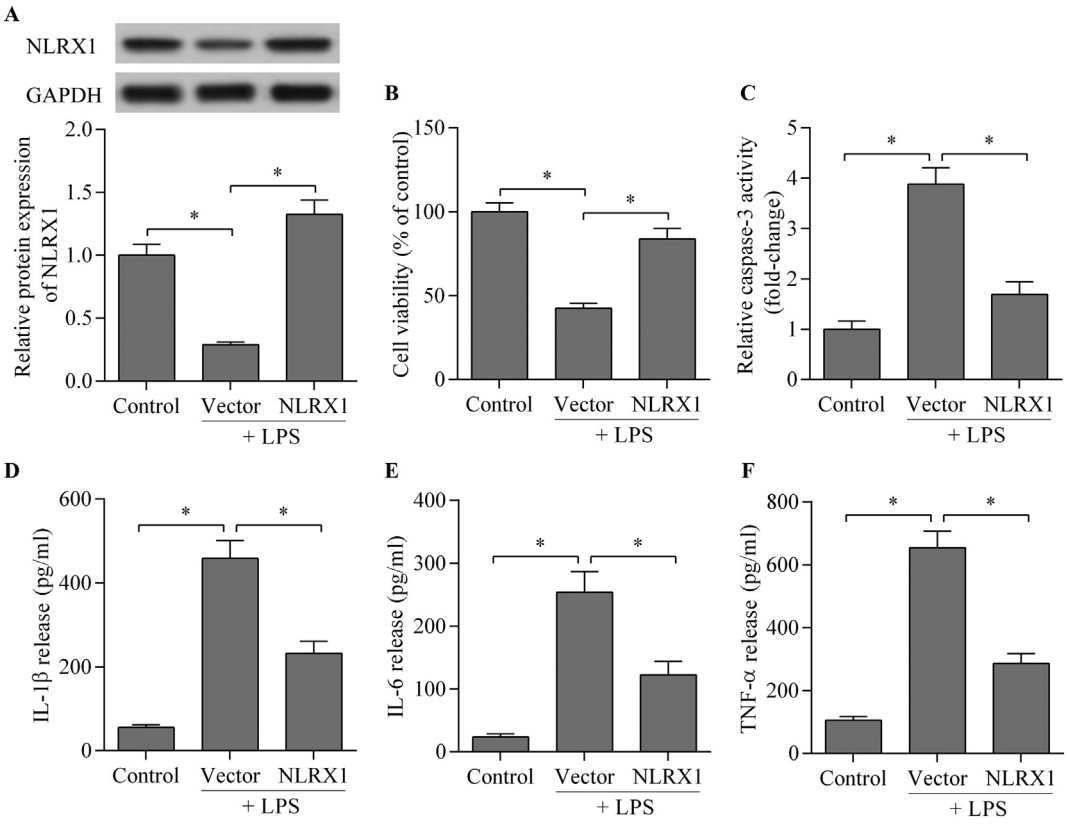

Fig. 2. NLRX1 overexpression alleviated LPS-induced apoptosis and inflammation in chondrocytes. Chondrocytes were transfected with pcDNA3.1/NLRX1 vector (NLRX1) or pcDNA3.1 empty vector (vector) for 48 h and then exposed to LPS for 6 h. (A) NLRX1 protein expression was examined by western blot. (B) Cell viability was measured with the MTT assay. (C) Cell apoptosis was determined by measuring caspase-3 activity. The release of (D) IL-1β, (E) IL-6, and (F) TNF-α was detected by ELISA kits. *$p < 0.05$.

measured with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) in accordance with the manufacturer's protocols.

2.9. Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using SPSS Statistics Version 19.0 (SPSS Inc., Chicago, IL, USA). Comparisons among multiple groups were determined by one-way analysis of variance followed by a *post hoc* Bonferroni test. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. NLRX1 expression was decreased in chondrocytes exposed to LPS

To investigate whether NLRX1 is involved in cartilage inflammation in OA, we measured the expression of NLRX1 in chondrocytes stimulated with LPS *in vitro*. qPCR revealed that NLRX1 mRNA expression was significantly downregulated in chondrocytes after LPS treatment compared with untreated cells (Fig. 1A). Consistently, LPS treatment markedly downregulated the NLRX1 protein expression in chondrocytes (Fig. 1B). These data indicate that LPS treatment decreases NLRX1 expression in chondrocytes.

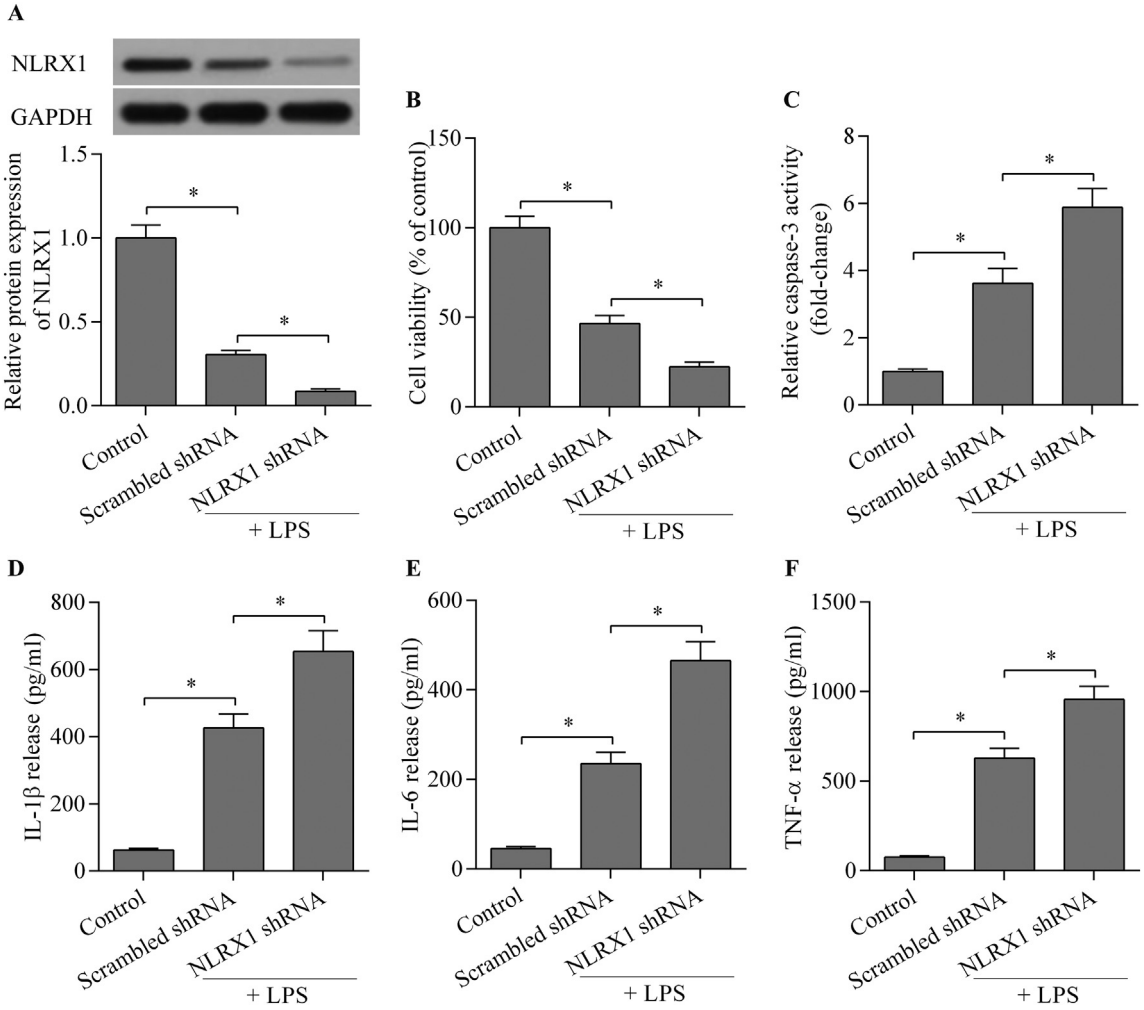

Fig. 3. NLRX1 knockdown exacerbated LPS-induced apoptosis and inflammation in chondrocytes. Chondrocytes were transfected with NLRX1 or scrambled shRNA for 48 h and then treated with LPS for 6 h. (A) NLRX1 protein expression was detected by western blot. (B) The MTT assay was performed to determine cell viability. (C) Cell apoptosis was assessed by measuring caspase-3 activity. The release of (D) IL-1β, (E) IL-6, and (F) TNF-α was measured by ELISA kits. $*p < 0.05$.

3.2. NLRX1 overexpression alleviated LPS-induced apoptosis and inflammation in chondrocytes

LPS stimulation evoked high levels of apoptosis and pro-inflammatory cytokine release in chondrocytes (Fig. 2A–F). To investigate whether NLRX1 regulates LPS-induced apoptosis and inflammation in chondrocytes, we performed gain-of-function experiments for NLRX1 by using an NLRX1 expression vector. Transfection of the NLRX1 expression vector in chondrocytes significantly upregulated NLRX1 expression (Fig. 2A). NLRX1 overexpression significantly improved chondrocyte viability after LPS stimulation (Fig. 2B). The caspase-3 activity assay showed that LPS caused high caspase-3 activity that was significantly decreased by NLRX1 overexpression (Fig. 2C). Moreover, we evaluated the effect of NLRX1 overexpression on LPLS-induced inflammation; overexpression significantly downregulated IL-1β, IL-6, and TNF-α release in LPS-stimulated chondrocytes (Fig. 2D–F). Collectively, these results suggest that NLRX1 overexpression alleviates LPS-induced apoptosis and inflammation in chondrocytes.

3.3. NLRX1 silencing exacerbated LPS-induced apoptosis and inflammation in chondrocytes

To verify that NLRX1 participates in regulating LPS-stimulated chondrocytes, we performed loss-of-function experiments by silencing NLRX1. Transfection with an NLRX1 shRNA expression vector significantly decreased NLRX1 expression in chondrocytes (Fig. 3A). As expected, our results revealed that NLRX1 silencing significantly reduced chondrocyte viability following LPS treatment and exacerbated LPS-induced apoptosis (Fig. 3B and C). Moreover, NLRX1 silencing increased IL-1β, IL-6 and TNF-α release from chondrocytes stimulated with LPS (Fig. 3D–F). Together, these data indicate that NLRX1 inhibition worsens LPS-induced apoptosis and inflammation in chondrocytes.

3.4. NLRX1 regulated NF-κB signaling activation in LPS-treated chondrocytes

To uncover the molecular mechanism that underlies the NLRX1-regulated effects on LPS-stimulated chondrocytes, we examined the regulatory effect of NLRX1 on NF-κB signaling. NLRX1 overexpression significantly decreased the phosphorylation of IKKβ and NF-κB p65 (Fig. 4A and B). Moreover, a luciferase reporter assay showed that NLRX1 overexpression significantly deactivated NF-κB signaling in LPS-stimulated chondrocytes (Fig. 4C). By contrast, NLRX1 silencing had the opposite effect (Fig. 4D–F). Overall, these results confirmed that NLRX1 regulates LPS-induced NF-κB activation in chondrocytes.

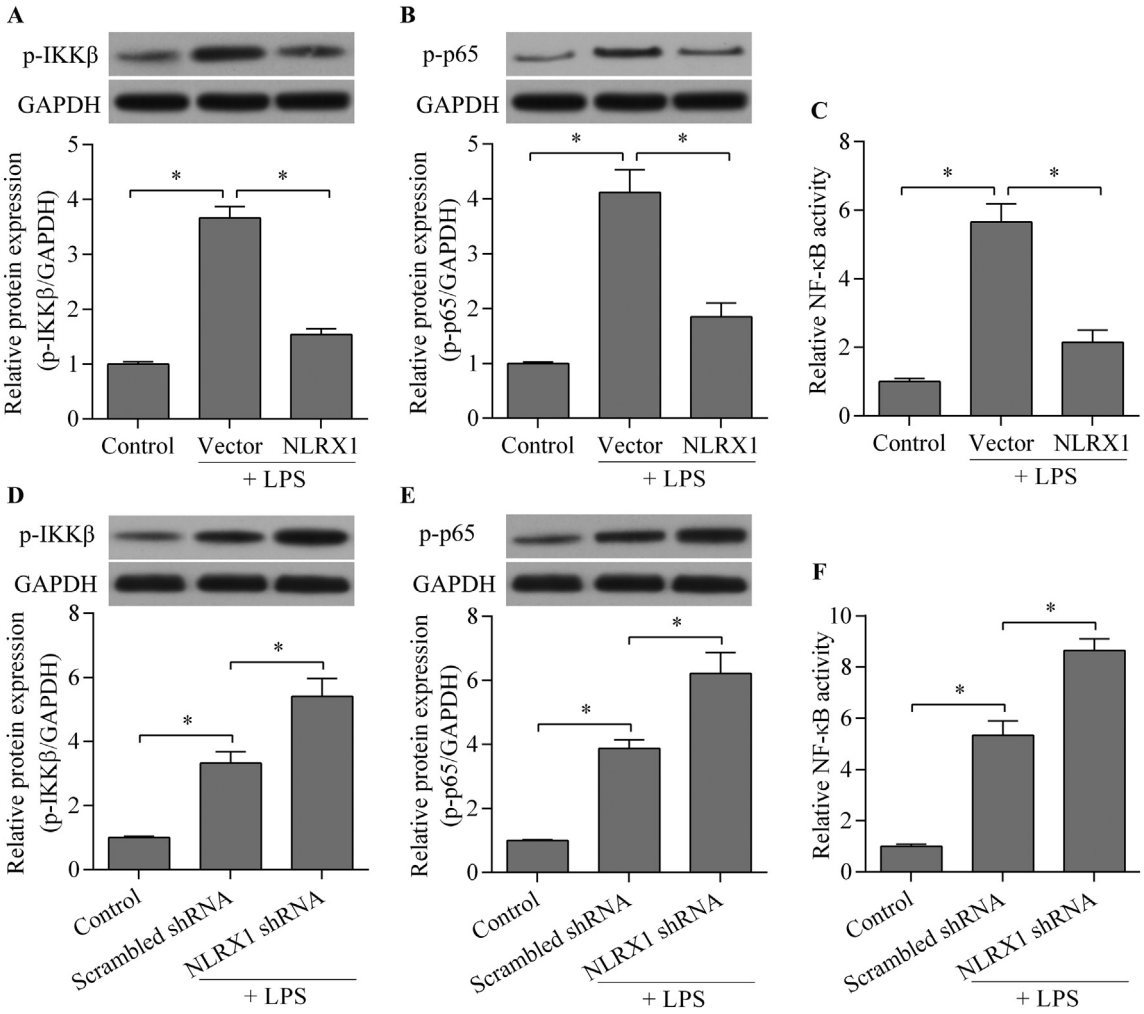

Fig. 4. NLRX1 regulated NF-κB activation in LPS-stimulated chondrocytes. The effect of NLRX1 overexpression on protein expression of (A) p-IKKβ and (B) NF-κB p-p65 was detected by western blot. (C) The effect of NLRX1 overexpression on LPS-induced NF-κB activation was examined by a luciferase reporter assay. The effect of NLRX1 silencing on protein expression of (D) p-IKKβ and (E) NF-κB p-p65 was examined by western blot. (F) The effect of NLRX1 silencing on LPS-induced NF-κB activation was assessed by a luciferase reporter assay. *$p < 0.05$.

3.5. NF-κB inhibition reversed the enhancing effect of NLRX1 silencing on LPS-induced apoptosis and inflammation

To investigate whether NLRX1 regulates LPS-induced apoptosis and inflammation through NF-κB signaling, we evaluated the effect of NF-κB inhibition on the NRLX1-silencing-mediated effect in LPS-stimulated chondrocytes. Treatment with the NF-κB inhibitor PTDC significantly decreased NF-κB activation in LPS-treated chondrocytes (Fig. 5A). As expected, the enhancing effect of NLRX1 silencing on LPS-induced apoptosis and inflammation was significantly reversed by NF-κB inhibition (Fig. 5B–F). These data indicate that NLRX1 participates in LPS-induced apoptosis and inflammation in chondrocytes by regulating NF-κB signaling.

4. Discussion

In the present study, our results revealed an important role of NLRX1 in regulating LPS-induced apoptosis and inflammation in chondrocytes *in vitro*. We found that NLRX1 expression was decreased in chondrocytes treated with LPS, and NLRX1 overexpression significantly alleviated LPS-induced apoptosis and inflammation. We further determined that the underlying mechanism was associated with its regulatory effect on NF-κB signaling activation (Fig. 6). Our study suggests a potential role of NLRX1-mediated NF-κB signaling in the progression of OA.

NLRX1 inhibits ischemia-reperfusion-injury-induced apoptosis, associated with preventing oxidative stress and sustaining mitochondrial activity [27]. NLRX1 restoration impedes cigarette-smoke-induced cell death and apoptosis in a mouse model of chronic obstructive pulmonary disease [34]. NLRX1 prevents influenza-virus-induced apoptosis and preserves the antiviral function of macrophages [35]. Although these findings indicate an anti-apoptotic function for NLRX1, a pro-apoptotic function for NRLX1 has also been reported. Yin et al. reported that NLRX1 accelerates cisplatin-induced apoptosis in cochlear cells by enhancing reactive oxygen species (ROS) production [36,37]. Moreover, NLRXL1 functions as a tumor suppressor that promotes cancer cell apoptosis [19,38]. These findings suggest that the precise role of NLRX1 in regulating apoptosis may be dependent on different cell types and stimulations. Herein, our results demonstrated that NLRX1 overexpression inhibited LPS-induced apoptosis in chondrocytes, a finding that supports an anti-apoptotic role for NLRX1. Therefore, the precise role of NLRX1 in regulating apoptosis requires further study using different cell types under different stimulations.

NLRX1 plays an important role in various inflammation-related pathological processes [39]. NLRX1 knockout exacerbates tissue damage in mice with experimental autoimmune encephalomyelitis; this effect is associated with enhanced microglial inflammation in the nervous system [40]. NLRX1 expression is decreased in a mouse model of

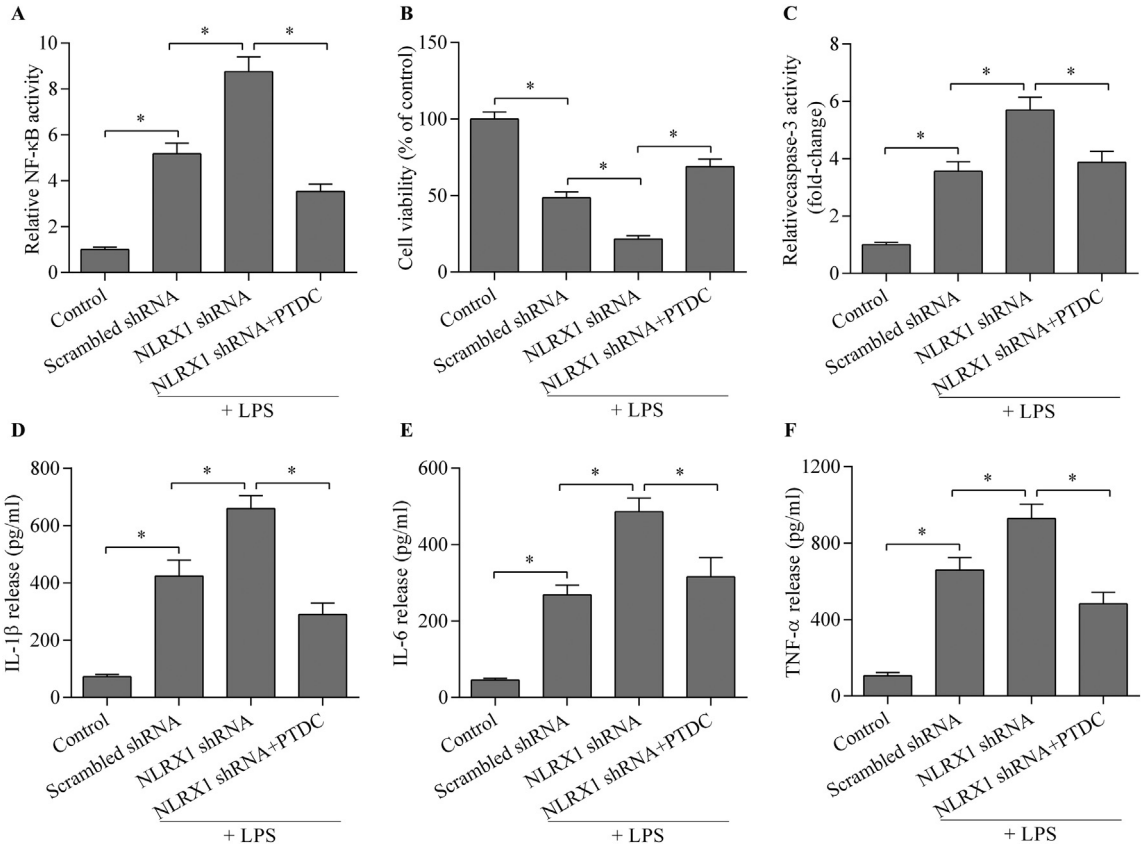

Fig. 5. NF-κB inhibition reversed the enhancing effect of NLRX1 silencing on LPS-induced apoptosis and inflammation. Chondrocytes were transfected with NLRX1 or scrambled shRNA for 48 h in the presence of 50 μM PTDC and then treated with LPS for 6 h. (A) NF-κB activity was detected by a luciferase reporter assay. (B) Cell viability was determined by the MTT assay. (C) Cell apoptosis was detected by measuring caspase-3 activity. The release of (D) IL-1β, (E) IL-6, and (F) TNF-α was measured by ELISA kits. *$p < 0.05$.

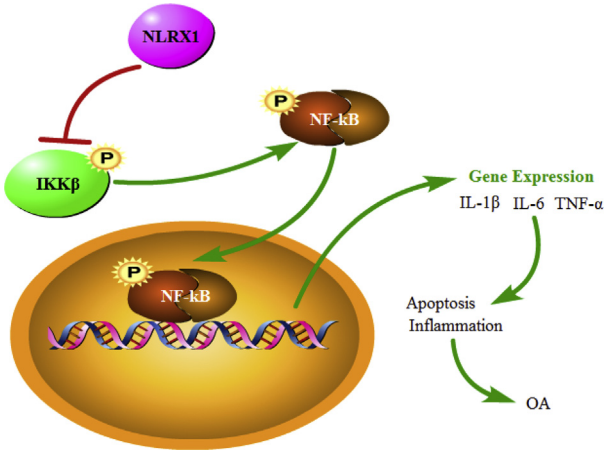

Fig. 6. Schematic model of NLRX1-mediated NF-κB signaling in regulating LPS-induced apoptosis and inflammation in chondrocytes.

nonalcoholic steatohepatitis, and this change indicates the potential relevance for NLRX1 in the development of this pathology [41]. NLRX1 expression is downregulated in lung tissues of chronic obstructive pulmonary disease, and NLRX1 deficiency augments cigarette smoke-induced inflammation in a mouse model [34]. Notably, NLRX1 overexpression alleviates myocardial ischemia-induced inflammatory responses and plays a cardioprotective role in myocardial ischemic injury [28]. These findings suggest that NLRX1 is an anti-inflammatory factor. Consistently, our findings demonstrated that NLRX1 overexpression suppressed LPS-induced inflammation in chondrocytes, while NLRX1 knockdown augmented LPS-induced inflammation in chondrocytes; these results support an anti-inflammatory function for NLRX1 in LPS-induced inflammation. Our findings are consistent with a recent study by Xia et al., which showed that NLRX1 restricts LPS-induced inflammation in various cell types [30]. Our study reveals, for the first time, an important role for NLRX1 in chondrocyte inflammation and suggests a potential role of NLRX1 in OA.

Unrestricted NF-κB signaling is an important event in the pathophysiology of inflammation-related diseases, including OA. Interestingly, NLRX1 has emerged as a crucial regulator of NF-κB signaling. Xia et al. reported that NLRX1 suppresses LPS-induced NF-κB signaling activation by interacting with IKK proteins [30]. NLRX1 decreases IKKα/β phosphorylation and blocks NF-κB signaling activation, actions which downregulate the expression of pro-inflammatory cytokines [30]. Consistent with these results, Allen et al. reported that NLRX1 attenuates influenza-virus- and LPS-induced inflammatory responses through restricting NF-κB activation. Loss of NLRX1 exacerbates neural tissue damage following brain injury associated with enhanced NF-κB signaling [29]. Moreover, NLRX1 suppresses tumorigenesis by negatively regulating NF-κB signaling [42]. In this study, NLRX1 overexpression downregulated phosphorylation of IKKβ and NF-κB p65 proteins and thus impeded LPS-induced NF-κB activation in chondrocytes. Our findings support NLRX1 as a negative regulator of NF-κB. Notably, we demonstrated that inhibition of NF-κB activation significantly reversed NLRX1-inhibition-induced promotion of LPS-induced inflammation, and this finding indicates that NLRX1 regulates LPS-induced inflammation in chondrocytes through NF-κB signaling. Collectively, combining our results with previous findings raises the possibility that NLRX1 may serve as a conceivable target for modulating NF-κB signaling, and this possibility suggests its potential

application in the treatment of inflammatory diseases.

Our present study has certain limitations. While our results demonstrated that NLRX1 regulates LPS-induced apoptosis and inflammation in chondrocytes through NF-κB signaling, these data were mainly obtained using an *in vitro* cellular model. Whether NLRX1 participates in OA pathology *in vivo* remains unclear. Therefore, the precise role of NLRX1/NF-κB axis in OA requires further investigation using animal models.

In conclusion, our study demonstrates that NLRX1 alleviates LPS-induced apoptosis and inflammation in chondrocytes by suppressing NF-κB signaling activation, a finding that highlights the potential relevance of NLRX1 in OA. However, the precise role of NLRX1/NF-κB signaling in OA pathology requires further investigation using *in vivo* animal models. Understanding such potential NLRX1-mediated molecular mechanisms might be vital for validating NLRX1 as a novel and promising target for OA treatment.

Conflict of interest

The authors declare that they have no conflict of interest.

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